



**QUEEN'S  
UNIVERSITY  
BELFAST**

## **Antibody-induced internalization of the human respiratory syncytial virus fusion protein**

Leemans, A., De Schryver, M., Van der Gucht, W., Heykers, A., Pintelon, I., Hotard, A. L., Moore, M. L., Melero, J. A., McLellan, J. S., Graham, B., Broadbent, L., Power, U. F., Caljon, G., Cos, P., Maes, L., & Delputte, P. (2017). Antibody-induced internalization of the human respiratory syncytial virus fusion protein. *Journal of Virology*. <https://doi.org/10.1128/JVI.00184-17>

**Published in:**  
Journal of Virology

**Document Version:**  
Peer reviewed version

**Queen's University Belfast - Research Portal:**  
[Link to publication record in Queen's University Belfast Research Portal](#)

### **Publisher rights**

© 2017 American Society for Microbiology. This work is made available online in accordance with the publisher's policies. Please refer to any applicable terms of use of the publisher.

### **General rights**

Copyright for the publications made accessible via the Queen's University Belfast Research Portal is retained by the author(s) and / or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights.

### **Take down policy**

The Research Portal is Queen's institutional repository that provides access to Queen's research output. Every effort has been made to ensure that content in the Research Portal does not infringe any person's rights, or applicable UK laws. If you discover content in the Research Portal that you believe breaches copyright or violates any law, please contact [openaccess@qub.ac.uk](mailto:openaccess@qub.ac.uk).

1 **Antibody-induced internalization of the human respiratory syncytial virus**  
2 **fusion protein**

3 Leemans A<sup>1</sup>, De Schryver M<sup>1</sup>, Van der Gucht W<sup>1</sup>, Heykers A<sup>1</sup>, Pintelon I<sup>2</sup>, Hotard AL<sup>3</sup>, Moore  
4 ML<sup>3,4</sup>, Melero JA<sup>5</sup>, McLellan JS<sup>6</sup>, Graham BS<sup>7</sup>, Broadbent L<sup>8</sup>, Power UF<sup>8</sup>, Caljon G<sup>1</sup>, Cos P<sup>1</sup>,  
5 Maes L<sup>1</sup>, Delpitte P<sup>1#</sup>

6  
7 <sup>1</sup> Laboratory of Microbiology, Parasitology and Hygiene, University of Antwerp, Antwerp,  
8 Belgium

9 <sup>2</sup> Laboratory of Cell Biology and Histology, University of Antwerp, Antwerp, Belgium

10 <sup>3</sup> Department of Pediatrics, Emory University School of Medicine, Atlanta, Georgia, United  
11 States of America

12 <sup>4</sup> Children's Healthcare of Atlanta, Atlanta, Georgia, United States of America

13 <sup>5</sup> Centro Nacional de Microbiología and CIBER de Enfermedades Respiratorias, Instituto de  
14 Salud Carlos III, Majadahonda, Madrid, Spain

15 <sup>6</sup> Department of Biochemistry, Geisel School of Medicine at Dartmouth, Hanover, New  
16 Hampshire, United States of America

17 <sup>7</sup> Vaccine Research Center, National Institute of Allergy and Infectious Diseases, National  
18 Institutes of Health, Bethesda, Maryland, United States of America

19 <sup>8</sup> Centre for Infection & Immunity, School of Medicine, Dentistry & Biomedical Sciences,  
20 Queen's University Belfast, Belfast, United Kingdom

21 <sup>#</sup>Corresponding author. Laboratory of Microbiology, Parasitology and Hygiene, University of  
22 Antwerp, Universiteitsplein 1, 2610 Antwerpen, Belgium

23 Tel: +32 3 265 26 25

24 E-mail address: peter.delpitte@uantwerpen.be

- 25    **Running title:** Antibody-induced RSV F protein internalization
- 26    **Abstract:** 392 words
- 27    **Text:** 5030 words

**ABSTRACT**

Respiratory syncytial virus (RSV) infections remain a major cause of respiratory disease and hospitalizations among infants. Infection recurs frequently and establishes a weak and short-lived immunity. To date, RSV immunoprophylaxis and vaccine research is mainly focused on the RSV fusion (F) protein, but a vaccine remains elusive. The RSV F protein is a highly conserved surface glycoprotein and the main target of neutralizing antibodies induced by natural infection. Here, we analyzed an internalization process of antigen–antibody complexes after binding of RSV-specific antibodies to RSV antigens expressed on the surface of infected cells. The RSV F protein and attachment (G) protein were found to be internalized in both infected and transfected cells after the addition of either RSV-specific polyclonal antibodies (pAbs) or RSV glycoprotein-specific monoclonal antibodies (mAbs), as determined by indirect immunofluorescence staining and flow-cytometric analysis. Internalization experiments with different cell lines, well-differentiated primary bronchial epithelial cells (WD-PBECs) and RSV isolates suggest that antibody-internalization can be considered as a general feature of RSV. More specifically for RSV F, the mechanism of internalization was shown to be clathrin-dependent. All RSV F-targeted mAbs tested, regardless of their epitopes, induced internalization of RSV F. No differences could be observed between the different mAbs, indicating that RSV F internalization was epitope-independent. Since this process can be either antiviral, by affecting virus assembly and production, or beneficial for the virus, by limiting the efficacy of antibodies and effector mechanism, further research is required to determine the extent to which this occurs *in vivo* and how this might impact RSV replication.

52 **IMPORTANCE**

53

54 Current research into the development of new immunoprophylaxis and vaccines is mainly  
55 focused on the RSV F protein since, among others, RSV F-specific antibodies are able to protect  
56 infants from severe disease, if administered prophylactically. However, antibody responses  
57 established after natural RSV infections are poorly protective against reinfection and high levels  
58 of antibodies do not always correlate with protection. Therefore, RSV might be capable of  
59 interfering, at least partially, with antibody-induced neutralization. In this study, a process  
60 through which surface-expressed RSV F proteins are internalized after interaction with RSV-  
61 specific antibodies is described. On the one hand, this antigen-antibody complex internalization  
62 could result in an antiviral effect, since it may interfere with virus particle formation and virus  
63 production. On the other hand, this mechanism may also reduce the efficacy of antibody-  
64 mediated effector mechanisms towards infected cells.

## INTRODUCTION

Human respiratory syncytial virus (RSV) is a leading cause of severe lower respiratory tract disease in young children and a major cause in the elderly and immunocompromised patients worldwide (1, 2). Nearly all children are exposed to RSV by two years of age, and prematurity, bronchopulmonary dysplasia and congenital heart disease are risk factors for developing severe RSV disease, including bronchiolitis and pneumonia (1). RSV may also cause significant disease in adults and furthermore re-infection can occur throughout life (2). Despite the discovery of the virus in 1956, no safe and effective vaccine is currently available to control RSV infections (3). Treatment of severe infections is primarily supportive by maintenance of hydration and oxygenation. Palivizumab, a humanized monoclonal antibody, targets a conserved epitope of the RSV fusion (F) protein and is administered prophylactically to high-risk patients (4). Severe RSV disease appears to be linked to an unbalanced and incomplete immune response. Several factors that allow RSV to evade host defense have already been described (2, 5, 6).

RSV belongs to the Pneumoviridae, genus Orthopneumovirus, which is comprised of enveloped viruses with a negative-stranded RNA genome. The 15.2 kb non-segmented genome is comprised of 10 genes that encode 11 proteins. Among these are three surface glycoproteins, the G glycoprotein, the F protein and the small hydrophobic (SH) protein (1). The G protein is responsible for attachment with host cells, which are predominantly ciliated airway epithelial cells (7, 8). Fusion of the viral and cellular membranes is facilitated by the RSV F protein, as is fusion between the membranes of infected cells with adjacent cells, which result in large, multinucleated syncytia. The smaller SH protein is considered to act like a viroporin and increases membrane permeability (5). Of these envelope glycoproteins, only the RSV F protein is indispensable for viral replication *in vitro* (9). It is the most conserved RSV glycoprotein and also

89 the main target of neutralizing antibodies and vaccine development (10, 11). Initially, the RSV F  
90 protein assembles into a homotrimeric, metastable prefusion conformation that rearranges to a  
91 highly stable postfusion conformation during fusion of the viral and target cell membrane or  
92 spontaneously (12). Six major antigenic sites are currently identified that are located on the  
93 prefusion and/or postfusion trimer conformation of the RSV F protein (10, 13-15). Palivizumab,  
94 directed to antigenic site II, is the only approved immunoprophylaxis and provided a 55%  
95 reduction in RSV-associated hospitalizations in a phase III trial (16). At present, the use of potent  
96 neutralizing antibodies directed to other epitopes and/or targets is being extensively studied as  
97 alternative approaches for both therapy and prophylaxis. This research is mainly focused on  
98 highly potent antibodies that recognize the prefusion RSV F conformation. Three antibodies  
99 (5C4, AM22 and D25) were shown to bind the prefusion specific antigenic site Ø, located at the  
100 apex of the prefusion trimer (14). Recently, two novel prefusion-specific antibodies, MPE8 and  
101 AM14, were characterized and shown to bind antigenic sites III and V, respectively (10, 15, 17).  
102 The epitope for MPE8 is located near the binding site of palivizumab in the groove between the  
103 helix-turn-helix and the ridge of antigenic site IV on the adjacent protomer. It partially competes  
104 with mAbs to sites II, IV and V. This epitope is well-conserved between other pneumoviruses of  
105 the *Paramyxoviridae* family (15). Antigenic site V, targeted by AM14, spans from the tip of the  
106  $\beta 3$ - $\beta 4$  hairpin of one protomer to the distal end of antigenic site IV on the adjacent protomer (17).  
107 Internalization of viral envelope proteins expressed on the surface of infected cells is a  
108 commonly seen characteristic of viruses, including paramyxoviruses (18-22). For most viruses,  
109 the relevance of this process is not yet fully understood. In the case of the Henipavirus fusion  
110 proteins, internalization from the surface is essential for proteolytic activation by cathepsin L  
111 (19). Also, virus assembly can be affected by the internalization of viral glycoproteins (23).  
112 Furthermore, internalization can be important for viral pathogenesis by down-regulation of viral

113 antigen surface expression and reduced recognition of infected cells by the immune system (20,  
114 24-26). Two different types of internalization have been described previously. Spontaneous  
115 endocytosis was observed for many herpesviruses and human immunodeficiency virus (HIV)  
116 among others. A second type of internalization is induced by the interaction of specific antibodies  
117 with viral proteins expressed on the surface of infected cells, followed by internalization of  
118 antibody-antigen complexes in the cell (25, 27, 28). Such viral protein internalization may either  
119 result from cross-linking or depend on specific endocytic motifs in the cytoplasmic or  
120 transmembrane domains of glycoproteins, such as common tyrosine-based sorting motifs and di-  
121 leucine motifs (20, 24, 29, 30).

122 Previous studies have shown that upon binding of goat anti-RSV polyclonal antibodies (pAbs)  
123 to RSV antigens expressed on the surface of infected HEp-2 cells, internalization of these RSV  
124 antigen-antibody complexes may occur (31, 32). In this study, we examined this process in the  
125 context of a viral infection as well as at the level of individual RSV glycoproteins expressed at  
126 the surface of transfected cells. Both strategies demonstrated uptake of RSV antigen-antibody  
127 complexes in a time-dependent manner that resulted in a reduction of surface expressed RSV  
128 antigens. This process is modulating surface expression of RSV antigens and may affect  
129 induction of and recognition by RSV-specific antibodies. Since the RSV F protein elicits the most  
130 potent neutralizing antibodies and is currently the most interesting target for therapeutic and  
131 prophylactic purposes, this study aimed to characterize antibody-induced internalization of RSV  
132 antigens, in particular RSV F, in more detail.

133

## 134 MATERIAL AND METHODS

135

### 136 Cells, virus and antibodies



137 Human epidermoid carcinoma larynx cell line (HEp-2) and A549 cell line were obtained from  
138 ATCC. The cells were grown in Dulbecco's modified Eagle medium (DMEM) and Ham's F-12K  
139 (Kaighn's) medium respectively, both supplemented with 10% inactivated fetal bovine serum  
140 (iFBS) (Thermo Fisher Scientific). BSR T7/5 cells were a gift of K.K. Conzelmann (Max-von-  
141 Pettenhofer-Institut, Munich, Germany) and grown in Glasgow's minimal essential medium  
142 (GMEM) supplemented with 10% iFBS and 2% minimal essential amino acids (Thermo Fisher  
143 Scientific). Well-differentiated primary paediatric bronchial epithelial cell (WD-PBECs) culture  
144 was described previously (33). RSV reference strains A2, B1 and clinical isolate A1998/3-2 were  
145 obtained from the Biodefense and Emerging Infections Research Resources Repository (BEI  
146 resources) and propagated in HEp-2 cells. Recombinant RSV encoding the far-red fluorescent  
147 protein monomeric Katushka-2 (mKate2) was recovered as described previously (34).  
148 Commercially available goat anti-RSV pAb (Virostat), mouse anti-RSV F IgG (clone 131-2A;  
149 Millipore) and mouse anti-RSV G IgG (clone 131-2G; Millipore) were used as reference  
150 antibodies. Human reference antiserum was obtained from BEI resources (BEI NR-4020). A  
151 panel of RSV F-specific mAbs and their corresponding Fab fragment were provided by J.A.  
152 Melero, J.S McLellan, B.S. Graham and C.A.M. de Haan. Secondary antibodies donkey anti-goat  
153 IgG, chicken anti-mouse IgG and goat-anti human IgG, conjugated with Alexa Fluor (AF) 488 or  
154 555, obtained from Thermo Fisher Scientific and FITC-conjugated rabbit anti-human IgG  
155 (DAKO), were used to visualize the antigens.

156

#### 157 **Construction and expression of recombinant RSV proteins**

158 Synthesis of the RSV F and RSV G protein was performed by Genscript and delivered in pUC57,  
159 a commonly used plasmid for cloning. Restriction enzymes (New England Biolabs) were used to  
160 subclone the recombinant sequences in a mammalian expression vector pBudCE4.1 (Thermo

161 Fisher Scientific). Transfection of the resulting plasmids was performed with Lipofectamine 2000  
162 (Thermo Fisher Scientific) in Opti-MEM (Thermo Fisher Scientific) to obtain surface expression  
163 of recombinant RSV proteins. Briefly, BSR T7/5 cells were seeded on coverslips in 24-well  
164 plates to be confluent at the time of transfection. Plasmid DNA was mixed with Lipofectamine  
165 2000 and incubated at room temperature during 20 min. Next, the transfection complexes were  
166 added to the cells and incubated for 2 h at room temperature. Further incubation at 37°C was  
167 performed after adding complete GMEM for 6 h. Finally, the complexes were removed, replaced  
168 by complete GMEM and incubated overnight at 37°C.

169

#### 170 **Antibody-induced internalization assay**

171 Cells were seeded on coverslips in 24-well plates to be subconfluent or confluent at the time of  
172 infection or transfection respectively. RSV infection was performed by diluting the virus stock in  
173 basal growth medium and subsequently adding the virus suspension to the cells. After 2 h  
174 incubation at 37°C, the inoculum was removed and replaced by complete growth medium and  
175 further incubated at 37°C. Transfections were performed as described above. After 24 h  
176 incubation, RSV-infected or transfected cells were incubated with antibodies against RSV for  
177 1 h at 4°C to allow only attachment of the antibodies. To remove unbound antibody, cells were  
178 washed three times with growth medium, followed by incubation at 37°C to start the  
179 internalization process. After different time points, cells were fixed with 4% paraformaldehyde  
180 (Merck) and permeabilized with 0.5% Triton X-100 (Sigma-Aldrich). To visualize antigen–  
181 antibody complexes, cells were stained with appropriate secondary antibodies conjugated with  
182 AF 488, AF 555 or FITC. As a control, cells were fixed after the 4°C incubation (T0).

183

#### 184 **Flow-cytometric analysis of RSV internalization**

185 Surface expression after internalization was quantified by flow cytometry. HEp-2 and A549 cells  
186 were seeded in 6-well plates to be subconfluent after 24 h incubation at 37°C or kept in  
187 suspension in pre-coated glass vials (Sigmacote<sup>®</sup>) for 24 h. Infection of the cells was performed  
188 as described above. After 24 h, infected cells in 6-well plates were detached by incubation with 1  
189 mM EDTA (Sigma) during 30 min at 4°C and pelleted by centrifugation (210 x g, 10 min, 4°C).  
190 The pellet was resuspended in RPMI supplemented with 10% iFBS, then incubated with primary  
191 antibodies during 1 h at 4°C and washed to remove unbound antibodies. Cells were kept at 4°C as  
192 timepoint 0. The other samples cells were then shifted to 37°C by the addition of warm RPMI  
193 medium and further incubated at 37°C during 90 min. To stop internalization, cells were shifted  
194 to 4°C for 15 min. Afterwards, the cells were incubated with AF 488-conjugated secondary  
195 antibodies for 1 h at 4°C, washed with PBS and analyzed by flow cytometry with a  
196 FACSCalibur. Dead cells were excluded by staining the cells with LIVE/DEAD<sup>®</sup> fixable far-red  
197 dead cell stain (Thermo Fisher Scientific). Forward-scattered light (FSC), side-scattered light, the  
198 AF 488 (FL-1) and far-red fluorescence signal (FL-4) were stored for further analysis. Mean  
199 fluorescence intensity (MFI) was calculated from three independent repeats. The reduction in  
200 surface expression was calculated as follows:  $100 - \frac{[MFI_{90 \text{ min}} - MFI_{\text{background}}]}{[MFI_{0 \text{ min}} - MFI_{\text{background}}]} \times 100$  whereas  $MFI_{\text{background}}$  is the mean of the fluorescence signal of stained non-  
201 infected cells.  
202

203

#### 204 **Spontaneous endocytosis assay**

205 Infection of subconfluent HEp-2 cell cultures was performed as described above. Surface proteins  
206 of the infected cells were biotinylated using EZ-link Sulfo-NHS-SS-biotin (Thermo Fisher  
207 Scientific) at 4°C. Then, cells were incubated at 37°C to allow endocytosis to occur. As a control  
208 of antibody-induced internalization, one sample was incubated with RSV-specific antibodies.

209 Biotin was removed from surface proteins by the addition of cleavage buffer (60 mM L-  
210 glutathione, 75 mM NaCl, 10 mM EDTA, pH 7.5) for 30 min at 4°C. One sample was neither  
211 incubated nor reduced with cleavage buffer to determine the total amount of biotinylated proteins.  
212 The efficiency of biotin removal by cleavage buffer was determined by cleavage of a non-  
213 incubated sample. After lysis of the cells with RIPA lysis buffer (Millipore), biotinylated proteins  
214 were immunoprecipitated using Streptavidin Mag Sepharose (Sigma) and then separated by  
215 sodium dodecyl sulfate-polyacrylamide gel electrophoresis under non-reducing conditions. The  
216 presence of RSV F proteins was detected by transferring the gel to a PVDF membrane and  
217 subsequent incubation with RSV F specific mAb.

218

#### 219 **Internalization inhibition assays**

220 To inhibit antibody-induced internalization, two strategies were performed as previously reported  
221 (35). First, inhibitors were used in different concentrations before and during the internalization  
222 assay. Amantadine and nystatin, purchased from Sigma, and myristoylated dynamin inhibitory  
223 peptide (DIP), purchased from Tocris Bioscience, were diluted in their solvent prior to dilution in  
224 cell-culture medium. After 1 h pretreatment of the cells with the inhibitors at 37°C, fresh inhibitor  
225 was added to the cells together with antibodies to induce internalization. As a control for each  
226 drug, cells were incubated with the corresponding solvent of the inhibitor. Controls were applied  
227 to confirm the effectiveness of the drugs, including biotinylated transferrin for clathrin-mediated  
228 endocytosis and FITC-labelled BSA for caveolae-mediated endocytosis. The biotinylated  
229 transferrin was visualized with streptavidin-FITC. Second, inhibition of internalization was  
230 performed using plasmids encoding dominant negative (DN) proteins that inhibit clathrin-,  
231 caveolae- or dynamin-mediated endocytosis. These constructs and their wild-type (WT)  
232 counterparts were originally provided by A. Benmerah (36, 37), A. Helenius (38, 39) and M.

233 McNiven (40, 41) respectively and further modified as described before (42). Co-transfection of  
234 BSR T7/5 cells was performed by diluting equal amounts of DNA of the RSV F protein and the  
235 DN or WT protein. WT and DN dynamin 2(aa) with a C-terminal enhanced GFP (eGFP) tag,  
236 were used to inhibit dynamin-dependent endocytosis. To inhibit clathrin-mediated endocytosis,  
237 the DN mutant of the protein Eps15 (DIII), essential for the docking of adaptor protein-2 during  
238 assembly of clathrin-coated pits, was used (42). This EGFP-tagged mutant has a deletion at the  
239 Eps15 homology and coiled-coil domains. A construct with a supplementary deletion of the AP-  
240 2-binding site served as a negative control (D3Δ2). WT and DN caveolin-1 constructs were used  
241 for caveolae-mediated endocytosis.

242

#### 243 **Microscopic analysis**

244 All high-resolution images of monolayer cultures were obtained using Apotome 2 with a Axio  
245 Observer inverted microscope with a compact light source HXP 120C (Zeiss) and using a Nikon  
246 Eclipse Ti-E inverted microscope attached to a microlens-enhanced dual spinning disk confocal  
247 system (UltraVIEW VoX; PerkinElmer, Zaventem,Belgium) equipped with 405 and 488 nm  
248 diode lasers for excitation of blue and green fluorophores, respectively. The images were  
249 obtained with ZEN 2012 and Volocity 3D Image Analysis Software.WD-PBEC images were  
250 obtained with a Nikon eclipse 90i inverted microscope.

251

#### 252 **Statistical analysis**

253 Data are presented as means ( $\pm$ SD) of three independent repeats and were analyzed by a student's  
254 t-test using GraphPad Prism 6. P values <0.05 were considered statistically significant.

255

## 256 RESULTS

257

### 258 Antibody-induced internalization of cell surface-expressed RSV antigens

259 To evaluate antibody-induced internalization of RSV antigens, RSV-infected HEp-2 and A549  
260 cells were incubated with polyclonal goat anti-RSV IgG for 1 h and subsequently fixed and  
261 stained with AF488 donkey anti-goat IgG. At time point 0, membrane staining was observed for  
262 both cell types and none of the infected cells showed vesicles in their cytoplasm (Fig.1A). After 1  
263 h incubation at 37°C, multiple intracellular vesicles were present in the cytoplasm of the cells and  
264 a reduction in surface expression was observed. No intracellular vesicles were observed in non-  
265 infected cells. Isotype control antibodies and secondary antibodies only were used to confirm that  
266 the internalization process is specifically induced by the interaction of RSV-specific antibodies  
267 with RSV antigens (data not shown). In addition, the kinetics of antibody-induced internalization  
268 was analyzed. Fig.1B shows the kinetics of the percentage of cells positive for intracellular RSV  
269 antigen-antibody complexes, which reached a maximum at 60 min and 90 min for HEp-2 cells  
270 and A549 cells, respectively. Similar observations were made based on the number of  
271 internalized vesicles (Fig.1C). By flow-cytometric analysis, the surface bound antibodies were  
272 measured before and after induction of internalization to allow a more quantitative measurement  
273 of internalization (43, 44). For HEp-2 cells and A549 cells, the mean fluorescence intensity  
274 (MFI) of surface RSV proteins after the addition of antibodies was reduced to 44% and 40%,  
275 respectively, showing that internalization occurs, but that not all RSV proteins are internalized  
276 (Fig.1D). Taken together, antibody-induced internalization of RSV antigens is time-dependent,  
277 but not all molecules are internalized upon binding of pAbs. Addition of increasing  
278 concentrations of pAbs resulted in rising levels of internalized vesicles between 0.001 and 0.01

279 mg/ml, followed by a slower increase between 0.01 and 0.3 mg/ml (Fig. 1E). At a concentration  
280 of 1 mg/ml, the internalization efficiency decreased.

281 Two major antigenic subgroups (A and B) have been described for RSV. With regards to the  
282 RSV surface proteins F and G, an amino acid identity of 91% and 51%, respectively, exists  
283 between the subgroups (45). After infection with the RSV B1 reference strain, internalization of  
284 RSV-specific pAbs in complex with RSV surface antigens was also observed (Fig.2A).  
285 Additionally, a clinical strain (A1998/3-2) was evaluated and numerous intracellular vesicles  
286 were also observed, and surface expression of RSV proteins was reduced compared to time point  
287 0 (Fig.2A). These findings suggest that internalization of RSV surface antigens is a general  
288 feature of RSV. In addition to polyclonal goat anti-RSV antibodies obtained after immunization,  
289 a human anti-RSV reference serum (obtained after natural RSV infection) was also evaluated.  
290 After a 60 min incubation at 37°C, internalization and antigen–antibody complexes were detected  
291 in the cell cytoplasm, and some complexes remained at the surface of infected HEp-2 cells  
292 (Fig.2B). Finally, the internalization process was also analyzed in RSV-infected well-  
293 differentiated primary bronchial epithelial cells (WD-PBECs). After 120 min incubation with  
294 human RSV-specific antiserum, internalized RSV antigen–antibody complexes were observed in  
295 more than 80% of the RSV-infected cells (Fig.3A,B).

296

#### 297 **Determination of the cell-surface expressed RSV proteins involved in the internalization** 298 **process**

299 Since RSV F and G proteins are the two major surface antigens and the only RSV proteins that  
300 induce neutralizing antibodies, the involvement of the respective RSV surface glycoproteins was  
301 identified. For this purpose, RSV glycoprotein-specific mAbs were used. For both surface  
302 proteins and in both cell types, internalization and intracellular vesicles were observed (Fig.4A).

303 Analysis of internalization in BSR T7/5 cells transfected with the individual RSV F or G proteins  
304 was performed to investigate their respective roles in this process. Internalization of RSV  
305 antigens was also observed after RSV infection of BSR T7/5 cells and incubation with RSV-  
306 specific antibodies, showing that this process also occurs in these cells (data not shown). For both  
307 proteins, multiple internalized antigen–antibody complexes were present in vesicles in the  
308 cytoplasm of cells transfected with the RSV F or G protein and after incubation with either RSV-  
309 specific pAbs or RSV glycoprotein-specific mAbs (Fig.4B). These observations show that  
310 internalization of both RSV surface proteins occurs independent of other viral proteins.

311

312 **Internalization of surface-expressed RSV F proteins is clathrin-mediated and mainly**  
313 **triggered after antibody-induced cross-linking of the RSV F protein**

314 As mentioned earlier, RSV F is the major target of neutralizing antibodies and plays a central role  
315 in the development of new immunoprophylaxis and vaccine strategies. Therefore, the  
316 characteristics of RSV F internalization were studied in more detail. The endocytic route through  
317 which RSV F-antibody complexes are internalized, was investigated by using inhibitors that  
318 block different mechanisms of endocytosis. DIP is an inhibitor of the GTPase dynamin that  
319 blocks the binding of dynamin to amphiphysin (46). Addition of this peptide during  
320 internalization resulted in a reduction of approximately 50% of internalized vesicles (Fig.5A).  
321 Inhibition by dynasore, an inhibitor that blocks dynamin by disturbing the plasma membrane  
322 cholesterol homeostasis (47), resulted in similar reductions (data not shown). In addition, BSR  
323 T7/5 cells expressing a recombinant RSV F protein were co-transfected with dominant negative  
324 (DN) proteins to inhibit a specific internalization process. By using an eGFP-tagged DN mutant  
325 of dynamin 2(aa) in RSV F transfected BSR T7/5 cells, a significant reduction of internalized  
326 vesicles (62.01%) was observed in cells transfected with DN dynamin compared to WT dynamin



transfected cells (Fig.5B,G). Both results show the involvement of a dynamin-dependent mechanism. Since GTPase dynamin is recruited to both clathrin-coated pits and caveolae, further distinction was made between these endocytic routes (48). Amantadine was used to test the dependence of clathrin and resulted in a dose-dependent reduction of intracellular vesicles with a maximum of 74.09% (Fig.5C). By using a DN mutant of Eps 15 (DIII) and the control plasmid DIIIΔ2 the role of clathrin was further analyzed. DIII transfected cells showed a significant reduction in internalization compared to DIIIΔ2 transfected cells (Fig.5D,G). No difference in the amount of internalized vesicles was observed after treatment of the cells with nystatin, a sterol-binding agent which disrupts caveolae (Fig.5E). These results were confirmed in RSV F transfected cells co-transfected with a GFP-tagged DN mutant of caveolin-1, which showed no significant differences in internalization of RSV F compared to cells transfected with the control plasmid, an eGFP-tagged WT caveolin-1 (Fig.5F,G).

In addition, we investigated the extent to which spontaneous endocytosis occurs for RSV proteins by labelling the surface proteins of infected cells with a membrane impermeable biotinylation reagent, followed by an internalization assay. Cell surface biotinylation was efficient (Fig.6E) and the addition of cell-impermeable glutathione removed almost all biotin from the cell surface (Fig.6D). Antibody-induced internalization of biotinylated surface proteins resulted in protection from glutathione-mediated biotin cleavage. A difference in the amount of intracellular (biotinylated) RSV F proteins could be observed between internalization induced by antibodies (Fig.6C) and spontaneous endocytosis in the absence of antibodies (Fig.6B), confirming that the majority of RSV F internalization is triggered by antibodies.

Intact monoclonal IgG antibodies can cross-link surface-expressed antigens and stimulate their internalization (49, 50). To determine whether cross-linking of RSV F proteins is required for internalization, monovalent Fab fragments of RSV F-specific mAbs were compared with the

351 intact mAbs (Fig.7A). Internalization of RSV F proteins was significantly reduced when induced  
352 by the monovalent Fab fragments (Fig.7B), indicating that cross-linking plays a role in efficient  
353 RSV F internalization.

354

### 355 **Internalization of surface RSV F proteins is triggered by binding of different neutralizing** 356 **RSV F epitope-specific mAbs**

357 Different neutralizing epitopes on the RSV F protein have already been identified and mAbs  
358 directed against these epitopes are available (10, 13-15). Since mAbs recognizing different  
359 epitopes of the same protein could have different effects on internalization, the epitope-  
360 dependence of RSV F internalization was analyzed (51). The amount of internalized RSV F-mAb  
361 complexes was determined by flow-cytometric analysis. After shifting RSV-infected HEp-2 cells  
362 in suspension to 37°C, anti-RSV F mAbs attached to surface RSV F proteins were internalized  
363 and the amount of internalization was quantified by a reduction of surface expression. A  
364 reduction in MFI was seen for all mAbs, compared to the MFI of cells that were kept at 4°C and  
365 did not undergo internalization. The amount of internalization, as calculated by the reduction of  
366 surface fluorescence, ranged from 31 to 57% for mAb 131-2A and AM22, respectively. Overall,  
367 no major differences could be observed between prefusion RSV F-specific mAbs or mAbs that  
368 bound both pre- and postfusion RSV F (Table 1). The results also showed that not all RSV F  
369 molecules are internalized upon stimulation with the mAbs. These findings were confirmed by  
370 analysis with fluorescence microscopy where surface expression of RSV F was still observed  
371 after induction of internalization (data not shown).

372

### 373 **DISCUSSION**

374

375 Our results show the potential of RSV-specific antibodies and human sera from RSV-infected  
376 patients to trigger internalization of RSV antigens expressed on the surface of infected cells.  
377 These findings were clearly documented by confocal microscopic analysis and flow cytometry,  
378 and observed in different epithelial cell lines (HEp-2, A549 and BEAS-2B cells (52, 53). In  
379 addition, WD-PBEC cultures were used to confirm that this process also occurs in a more  
380 representative model of primary airway epithelial cells. The amount of intracellular vesicles  
381 increased with increasing time of incubation and reached a plateau after 60 to 90 min in  
382 monolayer cells. Furthermore, surface-expressed glycoproteins of different RSV strains,  
383 including both reference strains (A2 and B1) and a clinical isolate (A1998/3-2), were shown to be  
384 susceptible to antibody-induced internalization (54). Taken together, our findings suggest that the  
385 internalization process is a general feature of RSV-infected cells. Using inhibitors and plasmids  
386 encoding dominant-negative proteins of endocytic pathways, the internalization was shown to be  
387 clathrin-dependent. However, since complete inhibition of RSV F internalization was not  
388 achieved, other mechanisms might be involved or could be induced upon blocking a specific  
389 pathway (55). To exclude the latter possibility, combinations of inhibitors were tested but did not  
390 show any difference compared to treatment with a single inhibitor (data not shown) (56).

391 Additionally, it was shown that RSV F internalization is mainly triggered upon binding of  
392 antibodies and is not merely spontaneous internalization, a well-described feature of several  
393 paramyxoviruses (18, 19, 21, 23). The results of a biotin internalization assay showed  
394 internalization of RSV F proteins after Ab-triggering. Only a weak signal was observed in the  
395 absence of antibodies to induce internalization, which might be a consequence of membrane  
396 turnover or residual non-cleaved proteins. Interestingly, a reduction in the molecular weight of  
397 the RSV F protein was observed upon internalization. A possible explanation is that upon  
398 internalization, an event occurs which cleaves the disulphide bridge between F1 and F2 subunits.

399 This can be mediated by the presence of enzymes in the endo-lysosomal system that reduce  
400 disulphide bonds and would thus also cleave the F1-F2 bonds (57, 58). Since the mAb used in  
401 this experiment is specific for F1, this cleavage will result in detection of a lower molecular  
402 weight band on WB, corresponding to the F1 subunit. In addition to binding, antibody-induced  
403 cross-linking is most likely needed since we observed that RSV F protein-specific, monovalent  
404 Fab fragments were not efficient in inducing internalization. Furthermore, at the highest Ab  
405 concentration tested, internalization was induced less efficiently. This could indicate that with  
406 high antibody concentrations, cross-linking of RSV F proteins does not efficiently occur because  
407 every F protein is bound by a different antibody and thereby cross-linking induced internalization  
408 signals would be lost.

409 Previous work showed that internalization of surface-expressed viral glycoproteins can be  
410 influenced by interactions with other viral proteins expressed in infected cells. For the Suid  
411 herpesvirus I glycoproteins gB and gD, antibody-induced cross-linking was shown to be required  
412 for efficient internalization in infected monocytes (22). In contrast, viral core proteins of measles  
413 virus are known to regulate the expression of viral glycoproteins and may inhibit internalization  
414 during infection to promote virus assembly (18). In our work, internalization was observed in  
415 cells transfected with a single RSV protein as well as in RSV-infected cells, indicating that RSV  
416 F internalization occurs independently and is not affected by the expression of other viral  
417 proteins. How exactly RSV F activates internalization upon antibody binding is not clear. For  
418 some viruses, it was shown that internalization can depend on specific endocytic motifs in  
419 cytoplasmic and/or transmembrane domain of the viral protein, such as common tyr-based motifs  
420 and di-leu motifs (18, 29, 30, 59). For other viruses, it is less clear that internalization depends on  
421 specific motifs and internalization may result from cross-linking. Analysis of the cytoplasmic tail  
422 of the RSV F protein did not reveal any known amino acid motifs involved in internalization.

423 Furthermore, analysis of antibody-induced internalization using a mutant RSV F protein lacking  
424 the cytoplasmic tail, only resulted in approximately 30% reduction of internalization (data not  
425 shown). This suggests that for RSV, specific amino acid motifs are not involved, and that cross-  
426 linking is a major driver for internalization, consistent with our finding that monovalent Fab  
427 fragments cannot efficiently induce internalization.

428 Several neutralizing RSV F-specific mAbs are described (Table 1). For some mAbs, it is  
429 shown that neutralization results from blocking virus fusion with host cell membranes by fixing  
430 RSV F proteins in their prefusion conformation (14, 60). Interestingly, their neutralizing activity  
431 may not only be directed against virions but also against virus-infected cells since cell-to-cell  
432 fusion can be inhibited by mAbs like palivizumab and motavizumab (60). In this study, flow-  
433 cytometric analysis showed that all RSV F-specific mAbs tested had the ability to decrease  
434 surface expression of RSV F proteins, which may impact cell-to-cell fusion. While this  
435 internalization may not affect cell-to-cell fusion for mAbs that directly block the RSV F fusion  
436 activity, it can still affect cell-to-cell fusion indirectly for mAbs that do not interfere with the  
437 RSV F fusion activity. As previously shown, different epitope recognition by mAbs could change  
438 the internalization pattern of the target antigen (51). However, we observed no differences  
439 between the different mAbs, all specific for one of the 6 known RSV F epitopes. These findings  
440 suggest that the antibody-induced internalization process of RSV F is not epitope-dependent.

441 Reinfection with RSV can occur throughout life, indicating that RSV antibody responses only  
442 partially provide protection and only for a limited period of time (2, 61). Prophylactic  
443 palivizumab is able to prevent severe RSV-induced respiratory tract disease in most, but not in all  
444 patients. The cause of the partial failure of this immunoprophylaxis remains unknown, but could  
445 be attributed to variations in the dose of inoculum, the efficiency of Ab transfer to the airways  
446 and the size of the airways. The process we observed may also affect the activity of anti-F

antibodies if the internalization strongly decreases the amount of RSV-specific antibodies. Presumably, this decrease will not be sufficient to impair the neutralization of free virus particles. Furthermore, the process we observed may also result in an antiviral effect, since internalization of the F protein could interfere with the formation of virus particles and spreading of the virus would thus be restricted already early in infection. On the contrary, interference in effector-mediated destruction of virus-infected cells by this process is more likely. In HIV-1 and SIV-infected cells, internalization of Env proteins provided protection from elimination by antibody-dependent cell-mediated cytotoxicity. Based on these findings, it was suggested that the efficacy of antibody-based therapeutics and HIV-1 vaccines could be improved by disturbing Env internalization (63). For herpesviruses, a similar observation was made (25). Surface expressed virus proteins were internalized by addition of specific antibodies, and antibody-dependent complement-mediated cell lysis was reduced approximately by 50% in infected cells upon antibody-induced internalization (25). Our results showed a remarkable decrease of surface-expressed F proteins after internalization, yet a portion remained on the cell surface. In this regard, further research is needed to elucidate whether there is sufficient internalization to protect RSV-infected cells from antibody-mediated effector responses.

In conclusion, this study describes a mechanism by which the RSV proteins expressed on the surface of infected cells are removed from the cell surface, together with RSV-specific antibodies, by internalization. Whether this process affects the activity of RSV F-specific antibodies and also has consequences for the *in vivo* replication and immune response remains to be elucidated.

## ACKNOWLEDGMENTS

471 We thank BEI resources for providing RSV reference strains, clinical isolates and human RSV  
472 antiserum. We also thank Dr. K.K. Conzelmann for providing BSR T7/5 cells.  
473 This work was supported by DOCPRO BOF (University Research Fund). The UltraVIEW VoX  
474 spinning disk confocal microscope system was purchased with support of the Hercules  
475 Foundation (Hercules Type 1: AUHA 09/001).

476 REFERENCES  
477

- 478 1. **Borchers AT, Chang C, Gershwin ME, Gershwin LJ.** 2013. Respiratory syncytial  
479 virus--a comprehensive review. *Clin Rev Allergy Immunol* **45**:331-379.
- 480 2. **Collins PL, Graham BS.** 2008. Viral and host factors in human respiratory syncytial  
481 virus pathogenesis. *J Virol* **82**:2040-2055.
- 482 3. **Graham BS, Modjarrad K, McLellan JS.** 2015. Novel antigens for RSV vaccines. *Curr*  
483 *Opin Immunol* **35**:30-38.
- 484 4. **Johnson S, Oliver C, Prince GA, Hemming VG, Pfarr DS, Wang SC, Dormitzer M,**  
485 **O'Grady J, Koenig S, Tamura JK, Woods R, Bansal G, Couchenour D, Tsao E, Hall**  
486 **WC, Young JF.** 1997. Development of a humanized monoclonal antibody (MEDI-493)  
487 with potent in vitro and in vivo activity against respiratory syncytial virus. *J Infect Dis*  
488 **176**:1215-1224.
- 489 5. **Collins PL, Melero JA.** 2011. Progress in understanding and controlling respiratory  
490 syncytial virus: still crazy after all these years. *Virus Res* **162**:80-99.
- 491 6. **Openshaw PJ, Chiu C.** 2013. Protective and dysregulated T cell immunity in RSV  
492 infection. *Curr Opin Virol* **3**:468-474.
- 493 7. **Villenave R, Thavagnanam S, Sarlang S, Parker J, Douglas I, Skibinski G, Heaney**  
494 **LG, McKaigue JP, Coyle PV, Shields MD, Power UF.** 2012. In vitro modeling of  
495 respiratory syncytial virus infection of pediatric bronchial epithelium, the primary target  
496 of infection in vivo. *Proc Natl Acad Sci U S A* **109**:5040-5045.
- 497 8. **Guo-Parke H, Canning P, Douglas I, Villenave R, Heaney LG, Coyle PV, Lyons JD,**  
498 **Shields MD, Power UF.** 2013. Relative respiratory syncytial virus cytopathogenesis in  
499 upper and lower respiratory tract epithelium. *Am J Respir Crit Care Med* **188**:842-851.
- 500 9. **Karron RA, Buonagurio DA, Georgiu AF, Whitehead SS, Adamus JE, Clements-**  
501 **Mann ML, Harris DO, Randolph VB, Udem SA, Murphy BR, Sidhu MS.** 1997.  
502 Respiratory syncytial virus (RSV) SH and G proteins are not essential for viral replication  
503 in vitro: clinical evaluation and molecular characterization of a cold-passaged, attenuated  
504 RSV subgroup B mutant. *Proc Natl Acad Sci U S A* **94**:13961-13966.
- 505 10. **McLellan JS.** 2015. Neutralizing epitopes on the respiratory syncytial virus fusion  
506 glycoprotein. *Curr Opin Virol* **11**:70-75.
- 507 11. **Tan L, Coenjaerts FE, Houspie L, Viveen MC, van Bleek GM, Wiertz EJ, Martin**  
508 **DP, Lemey P.** 2013. The comparative genomics of human respiratory syncytial virus  
509 subgroups A and B: genetic variability and molecular evolutionary dynamics. *J Virol*  
510 **87**:8213-8226.
- 511 12. **Liang B, Surman S, Amaro-Carambot E, Kabatova B, Mackow N, Lingemann M,**  
512 **Yang L, McLellan JS, Graham BS, Kwong PD, Schaap-Nutt A, Collins PL, Munir S.**  
513 2015. Enhanced Neutralizing Antibody Response Induced by Respiratory Syncytial Virus  
514 Prefusion F Protein Expressed by a Vaccine Candidate. *J Virol* **89**:9499-9510.
- 515 13. **McLellan JS, Chen M, Chang JS, Yang Y, Kim A, Graham BS, Kwong PD.** 2010.  
516 Structure of a major antigenic site on the respiratory syncytial virus fusion glycoprotein in  
517 complex with neutralizing antibody 101F. *J Virol* **84**:12236-12244.
- 518 14. **McLellan JS, Chen M, Leung S, Graepel KW, Du X, Yang Y, Zhou T, Baxa U,**  
519 **Yasuda E, Beaumont T, Kumar A, Modjarrad K, Zheng Z, Zhao M, Xia N, Kwong**  
520 **PD, Graham BS.** 2013. Structure of RSV fusion glycoprotein trimer bound to a  
521 prefusion-specific neutralizing antibody. *Science* **340**:1113-1117.



- 522 15. **Corti D, Bianchi S, Vanzetta F, Minola A, Perez L, Agatic G, Guarino B, Silacci C,**  
523 **Marcandalli J, Marsland BJ, Piralla A, Percivalle E, Sallusto F, Baldanti F,**  
524 **Lanzavecchia A.** 2013. Cross-neutralization of four paramyxoviruses by a human  
525 monoclonal antibody. *Nature* **501**:439-443.
- 526 16. **Homaira N, Rawlinson W, Snelling TL, Jaffe A.** 2014. Effectiveness of Palivizumab in  
527 Preventing RSV Hospitalization in High Risk Children: A Real-World Perspective. *Int J*  
528 *Pediatr* **2014**:571609.
- 529 17. **Gilman MS, Moin SM, Mas V, Chen M, Patel NK, Kramer K, Zhu Q, Kabeche SC,**  
530 **Kumar A, Palomo C, Beaumont T, Baxa U, Ulbrandt ND, Melero JA, Graham BS,**  
531 **McLellan JS.** 2015. Characterization of a Prefusion-Specific Antibody That Recognizes a  
532 Quaternary, Cleavage-Dependent Epitope on the RSV Fusion Glycoprotein. *PLoS Pathog*  
533 **11**:e1005035.
- 534 18. **Moll M, Klenk HD, Herrler G, Maisner A.** 2001. A single amino acid change in the  
535 cytoplasmic domains of measles virus glycoproteins H and F alters targeting, endocytosis,  
536 and cell fusion in polarized Madin-Darby canine kidney cells. *J Biol Chem* **276**:17887-  
537 17894.
- 538 19. **Vogt C, Eickmann M, Diederich S, Moll M, Maisner A.** 2005. Endocytosis of the  
539 Nipah virus glycoproteins. *J Virol* **79**:3865-3872.
- 540 20. **Bu Z, Ye L, Vzorov A, Taylor D, Compans RW, Yang C.** 2004. Enhancement of  
541 immunogenicity of an HIV Env DNA vaccine by mutation of the Tyr-based endocytosis  
542 motif in the cytoplasmic domain. *Virology* **328**:62-73.
- 543 21. **Popa A, Carter JR, Smith SE, Hellman L, Fried MG, Dutch RE.** 2012. Residues in  
544 the hendra virus fusion protein transmembrane domain are critical for endocytic recycling.  
545 *J Virol* **86**:3014-3026.
- 546 22. **Favoreel HW, Nauwynck HJ, Van Oostveldt P, Pensaert MB.** 2000. Role of anti-gB  
547 and -gD antibodies in antibody-induced endocytosis of viral and cellular cell surface  
548 glycoproteins expressed on pseudorabies virus-infected monocytes. *Virology* **267**:151-  
549 158.
- 550 23. **Robach JG, Lamb RA.** 2010. Analysis of parainfluenza virus-5 hemagglutinin-  
551 neuraminidase protein mutants that are blocked in internalization and degradation.  
552 *Virology* **406**:189-201.
- 553 24. **Favoreel HW, Van Minnebruggen G, Van de Walle GR, Ficinska J, Nauwynck HJ.**  
554 2006. Herpesvirus interference with virus-specific antibodies: bridging antibodies,  
555 internalizing antibodies, and hiding from antibodies. *Vet Microbiol* **113**:257-263.
- 556 25. **Van de Walle GR, Favoreel HW, Nauwynck HJ, Pensaert MB.** 2003. Antibody-  
557 induced internalization of viral glycoproteins and gE-gI Fc receptor activity protect  
558 pseudorabies virus-infected monocytes from efficient complement-mediated lysis. *J Gen*  
559 *Virol* **84**:939-948.
- 560 26. **Marsh M, Pelchen-Matthews A.** 2000. Endocytosis in viral replication. *Traffic* **1**:525-  
561 532.
- 562 27. **Chesebro B, Wehrly K, Doig D, Nishio J.** 1979. Antibody-induced modulation of Friend  
563 virus cell surface antigens decreases virus production by persistent erythroleukemia cells:  
564 influence of the Rfv-3 gene. *Proc Natl Acad Sci U S A* **76**:5784-5788.
- 565 28. **Shiraki K, Daikoku T, Takemoto M, Yoshida Y, Suzuki K, Akahori Y, Okuno T,**  
566 **Kurosawa Y, Asano Y.** 2011. Neutralizing anti-gH antibody of Varicella-zoster virus  
567 modulates distribution of gH and induces gene regulation, mimicking latency. *J Virol*  
568 **85**:8172-8180.

- 569 29. **Ficinska J, Van Minnebruggen G, Nauwynck HJ, Bienkowska-Szewczyk K, Favoreel**  
570 **HW.** 2005. Pseudorabies virus glycoprotein gD contains a functional endocytosis motif  
571 that acts in concert with an endocytosis motif in gB to drive internalization of antibody-  
572 antigen complexes from the surface of infected monocytes. *J Virol* **79**:7248-7254.
- 573 30. **Van Minnebruggen G, Favoreel HW, Nauwynck HJ.** 2004. Internalization of  
574 pseudorabies virus glycoprotein B is mediated by an interaction between the YQRL motif  
575 in its cytoplasmic domain and the clathrin-associated AP-2 adaptor complex. *J Virol*  
576 **78**:8852-8859.
- 577 31. **Sarmiento RE, Tirado RG, Valverde LE, Gomez-Garcia B.** 2007. Kinetics of  
578 antibody-induced modulation of respiratory syncytial virus antigens in a human epithelial  
579 cell line. *Virol J* **4**:68.
- 580 32. **Gutierrez-Ortega A, Sanchez-Hernandez C, Gomez-Garcia B.** 2008. Respiratory  
581 syncytial virus glycoproteins uptake occurs through clathrin-mediated endocytosis in a  
582 human epithelial cell line. *Virol J* **5**:127.
- 583 33. **Broadbent L, Villenave R, Guo-Parke H, Douglas I, Shields MD, Power UF.** 2016. In  
584 Vitro Modeling of RSV Infection and Cytopathogenesis in Well-Differentiated Human  
585 Primary Airway Epithelial Cells (WD-PAECs). *Methods Mol Biol* **1442**:119-139.
- 586 34. **Hotard AL, Shaikh FY, Lee S, Yan D, Teng MN, Plemper RK, Crowe JE, Jr., Moore**  
587 **ML.** 2012. A stabilized respiratory syncytial virus reverse genetics system amenable to  
588 recombination-mediated mutagenesis. *Virology* **434**:129-136.
- 589 35. **De Schryver M LA, Pintelon I, Cappoen D, Maes L, Caljon G, Cos P, Delputte PL.**  
590 2016. Comparative analysis of the internalization of the macrophage receptor sialoadhesin  
591 in human and mouse primary macrophages and cell lines. *Immunobiology* **In press**.
- 592 36. **Benmerah A, Lamaze C, Begue B, Schmid SL, Dautry-Varsat A, Cerf-Bensussan N.**  
593 1998. AP-2/Eps15 interaction is required for receptor-mediated endocytosis. *J Cell Biol*  
594 **140**:1055-1062.
- 595 37. **Benmerah A, Bayrou M, Cerf-Bensussan N, Dautry-Varsat A.** 1999. Inhibition of  
596 clathrin-coated pit assembly by an Eps15 mutant. *J Cell Sci* **112 ( Pt 9)**:1303-1311.
- 597 38. **Kurzchalia TV, Dupree P, Parton RG, Kellner R, Virta H, Lehnert M, Simons K.**  
598 1992. VIP21, a 21-kD membrane protein is an integral component of trans-Golgi-  
599 network-derived transport vesicles. *J Cell Biol* **118**:1003-1014.
- 600 39. **Pelkmans L, Helenius A.** 2002. Endocytosis via caveolae. *Traffic* **3**:311-320.
- 601 40. **Cao H, Garcia F, McNiven MA.** 1998. Differential distribution of dynamin isoforms in  
602 mammalian cells. *Mol Biol Cell* **9**:2595-2609.
- 603 41. **Cao H, Thompson HM, Krueger EW, McNiven MA.** 2000. Disruption of Golgi  
604 structure and function in mammalian cells expressing a mutant dynamin. *J Cell Sci* **113 (**  
605 **Pt 11)**:1993-2002.
- 606 42. **Dewerchin HL, Cornelissen E, Van Hamme E, Smits K, Verhasselt B, Nauwynck**  
607 **HJ.** 2008. Surface-expressed viral proteins in feline infectious peritonitis virus-infected  
608 monocytes are internalized through a clathrin- and caveolae-independent pathway. *J Gen*  
609 *Virol* **89**:2731-2740.
- 610 43. **Audran R, Drenou B, Wittke F, Gaudin A, Lesimple T, Toujas L.** 1995.  
611 Internalization of human macrophage surface antigens induced by monoclonal antibodies.  
612 *J Immunol Methods* **188**:147-154.
- 613 44. **Delputte PL, Van Gorp H, Favoreel HW, Hoebeke I, Delrue I, Dewerchin H,**  
614 **Verdonck F, Verhasselt B, Cox E, Nauwynck HJ.** 2011. Porcine sialoadhesin

- (CD169/Siglec-1) is an endocytic receptor that allows targeted delivery of toxins and antigens to macrophages. *PLoS One* **6**:e16827.
45. **Johnson PR, Collins PL.** 1988. The fusion glycoproteins of human respiratory syncytial virus of subgroups A and B: sequence conservation provides a structural basis for antigenic relatedness. *J Gen Virol* **69** ( Pt 10):2623-2628.
46. **Grabs D, Slepnev VI, Songyang Z, David C, Lynch M, Cantley LC, De Camilli P.** 1997. The SH3 domain of amphiphysin binds the proline-rich domain of dynamin at a single site that defines a new SH3 binding consensus sequence. *J Biol Chem* **272**:13419-13425.
47. **Preta G, Cronin JG, Sheldon IM.** 2015. Dynasore - not just a dynamin inhibitor. *Cell Commun Signal* **13**:24.
48. **Henley JR, Krueger EW, Oswald BJ, McNiven MA.** 1998. Dynamin-mediated internalization of caveolae. *J Cell Biol* **141**:85-99.
49. **Nielsen UB, Kirpotin DB, Pickering EM, Drummond DC, Marks JD.** 2006. A novel assay for monitoring internalization of nanocarrier coupled antibodies. *BMC Immunol* **7**:24.
50. **Watanabe N, Nomura T, Takai T, Chiba T, Honjo T, Tsubata T.** 1998. Antigen receptor cross-linking by anti-immunoglobulin antibodies coupled to cell surface membrane induces rapid apoptosis of normal spleen B cells. *Scand J Immunol* **47**:541-547.
51. **Hazin J, Moldenhauer G, Altevogt P, Brady NR.** 2015. A novel method for measuring cellular antibody uptake using imaging flow cytometry reveals distinct uptake rates for two different monoclonal antibodies targeting L1. *J Immunol Methods* **423**:70-77.
52. **Fleming EH, Kolokoltsov AA, Davey RA, Nichols JE, Roberts NJ, Jr.** 2006. Respiratory syncytial virus F envelope protein associates with lipid rafts without a requirement for other virus proteins. *J Virol* **80**:12160-12170.
53. **Yan D, Weisshaar M, Lamb K, Chung HK, Lin MZ, Plemper RK.** 2015. Replication-Competent Influenza Virus and Respiratory Syncytial Virus Luciferase Reporter Strains Engineered for Co-Infections Identify Antiviral Compounds in Combination Screens. *Biochemistry* **54**:5589-5604.
54. **Stokes KL, Chi MH, Sakamoto K, Newcomb DC, Currier MG, Huckabee MM, Lee S, Goleniewska K, Pretto C, Williams JV, Hotard A, Sherrill TP, Peebles RS, Jr., Moore ML.** 2011. Differential pathogenesis of respiratory syncytial virus clinical isolates in BALB/c mice. *J Virol* **85**:5782-5793.
55. **Lakadamyali M, Rust MJ, Zhuang X.** 2004. Endocytosis of influenza viruses. *Microbes Infect* **6**:929-936.
56. **Vercauteren D, Vandenbroucke RE, Jones AT, Rejman J, Demeester J, De Smedt SC, Sanders NN, Braeckmans K.** 2010. The use of inhibitors to study endocytic pathways of gene carriers: optimization and pitfalls. *Mol Ther* **18**:561-569.
57. **Pillay CS, Elliott E, Dennison C.** 2002. Endolysosomal proteolysis and its regulation. *Biochem J* **363**:417-429.
58. **Arunachalam B, Phan UT, Geuze HJ, Cresswell P.** 2000. Enzymatic reduction of disulfide bonds in lysosomes: characterization of a gamma-interferon-inducible lysosomal thiol reductase (GILT). *Proc Natl Acad Sci U S A* **97**:745-750.
59. **Sauter MM, Pelchen-Matthews A, Bron R, Marsh M, LaBranche CC, Vance PJ, Romano J, Haggarty BS, Hart TK, Lee WM, Hoxie JA.** 1996. An internalization signal in the simian immunodeficiency virus transmembrane protein cytoplasmic domain

- modulates expression of envelope glycoproteins on the cell surface. *J Cell Biol* **132**:795-811.
60. **Huang K, Incognito L, Cheng X, Ulbrandt ND, Wu H.** 2010. Respiratory syncytial virus-neutralizing monoclonal antibodies motavizumab and palivizumab inhibit fusion. *J Virol* **84**:8132-8140.
61. **Bont L, Versteegh J, Swelsen WT, Heijnen CJ, Kavelaars A, Brus F, Draaisma JM, Pekelharing-Berghuis M, van Diemen-Steen Voorde RA, Kimpen JL.** 2002. Natural reinfection with respiratory syncytial virus does not boost virus-specific T-cell immunity. *Pediatr Res* **52**:363-367.
62. **Ye L, Bu Z, Vzorov A, Taylor D, Compans RW, Yang C.** 2004. Surface stability and immunogenicity of the human immunodeficiency virus envelope glycoprotein: role of the cytoplasmic domain. *J Virol* **78**:13409-13419.
63. **von Bredow B, Arias JF, Heyer LN, Gardner MR, Farzan M, Rakasz EG, Evans DT.** 2015. Envelope Glycoprotein Internalization Protects Human and Simian Immunodeficiency Virus-Infected Cells from Antibody-Dependent Cell-Mediated Cytotoxicity. *J Virol* **89**:10648-10655.
64. **Anderson LJ, Bingham P, Hierholzer JC.** 1988. Neutralization of respiratory syncytial virus by individual and mixtures of F and G protein monoclonal antibodies. *J Virol* **62**:4232-4238.
65. **Wu H, Pfarr DS, Johnson S, Brewah YA, Woods RM, Patel NK, White WI, Young JF, Kiener PA.** 2007. Development of motavizumab, an ultra-potent antibody for the prevention of respiratory syncytial virus infection in the upper and lower respiratory tract. *J Mol Biol* **368**:652-665.

687 **FIGURE LEGENDS**

688

689 **FIG 1 Antibody-induced internalization of RSV antigens.** (A) HEp-2 cells and A549 cells  
690 were infected with RSV A2 for 24 h and then incubated with a pAb goat anti-RSV antibody  
691 bound at 4°C and then shifted to 37°C for 1 h to induce internalization. Afterwards the cells were  
692 fixed, permeabilized and stained with AF 488 donkey anti-goat IgG (green). Nuclei were  
693 visualized with DAPI (blue). Images represent a single confocal z-section through the middle of  
694 the cell. (B) Kinetics of the percentage of cells with internalized RSV antigen–antibody  
695 complexes for the indicated time points. (C) Kinetics of the amount of internalized vesicles in  
696 positive cells for the indicated time points. (D) Flow-cytometric analysis of RSV internalization.  
697 RSV-infected HEp-2 and A549 cells were kept in suspension during the induction of  
698 internalization and staining of the cells. The level of the remaining surface expressed RSV  
699 antigens after internalization is expressed as MFI relative to T0. (E) Effect of increasing  
700 concentrations of pAb goat anti-RSV antibodies on internalization. For each antibody  
701 concentration, the amount of internalized vesicles was quantified in 50 positive cells. Data  
702 represents the means ( $\pm$  SD) of 3 independent repeats.

703 **FIG 2 Antibody-induced internalization of RSV antigens with different RSV strains and**  
704 **different serum.** (A) HEp-2 cells were infected with RSV reference strain B1 or the clinical  
705 strain A1998/3-2 during 24 h. Goat anti-RSV pAbs were bound at 4°C (0 min) and internalization  
706 was induced at 37°C (60 min). (B) Human reference antiserum was used to induce internalization  
707 in RSV A2 infected HEp-2 cells. The cells of A and B were fixed, permeabilized, stained with  
708 AF 488 donkey anti-goat IgG and donkey anti-human IgG (green) respectively, and stained with  
709 DAPI (blue).

710 **FIG 3 Antibody-induced internalization of RSV antigens in WD-PBECs.** WD-PBEC cultures  
711 were infected with RSV/A2 mKate (MOI=3) in duplicate. At 72 h post infection, cultures were  
712 incubated with human sera for 1 h at 4°C, washed and then transferred to 37°C. Cultures were  
713 fixed at 0, 90 and 120 min with 4% paraformaldehyde, permeabilized and incubated with anti-  
714 human IgG FITC secondary for 1 h at 37°C. Images for data presented in A-C were taken using a  
715 Nikon Eclipse 90i microscope. Images for D were taken with a Leica SP5 confocal microscope.  
716 (A) 100 mKate positive RSV-infected cells at each time point in duplicate examined for evidence  
717 of internalized antibody using. (B) Representative images at each time point blue (DAPI), red  
718 (mKate), green (anti-human IgG 488 antibody). (C) Mock-infected cells incubated with sera for  
719 90 or 120 min and stained with the anti-human IgG 488 antibody. (D) En face and corresponding  
720 orthogonal sections with blue (DAPI), green (anti-human IgG 488 antibody). The confocal  
721 settings and gain were kept constant for all conditions.

722 **FIG 4 Antibody-induced internalization of RSV surface glycoproteins F and G.** (A) RSV  
723 infection of HEp-2 and A549 cells during 24 h was followed by incubation of the cells with anti-  
724 RSV F (clone 131-2A) or anti-RSV G (clone 131-2G) specific mouse mAbs for 1 h at 37°C. (B)  
725 RSV F and RSV G transfected BSR T7/5 cells were incubated with pAb goat anti-RSV or the  
726 corresponding mAbs during 1 h. At the indicated time points, the cells were fixed, permeabilized  
727 and stained with appropriate AF 488-conjugated secondary antibodies (green) and DAPI (blue).  
728 Images were acquired by fluorescence microscopy.

729 **FIG 5 Effect of inhibitors and DN proteins on antibody-induced RSV F internalization.**  
730 RSV-infected HEp-2 cells were incubated with mAb 131-2A in the presence of different  
731 concentrations of endocytic inhibitors, DIP (A), amantadine (C) and nystatin (E). After 90 min  
732 incubation, cells were fixed, permeabilized and stained. The amount of intracellular vesicles was



733 quantified and expressed as percentage relative to the number of vesicles in the absence of the  
734 inhibitor. BSR T7/5 cells were co-transfected with both RSV F (red) and DN proteins (dynamin 2  
735 (B), Eps15 (DIII) (D) and caveolin-1 (F)) (green). For each DN protein, also a control construct  
736 was used (WT dynamin 2 (B), inactive Eps15 (D3Δ2) (D), WT caveolin-1 (F)). After induction  
737 of internalization and staining of the cells, the amount of internalized vesicles was determined by  
738 fluorescence microscopy. Data represents the mean ( $\pm$ SD) of three replicates. (\*,  $P < 0.05$ ).

739 **FIG 6 Western blot analysis of a biotin internalization assay of RSV F proteins.** RSV-  
740 infected and non-infected HEp-2 cells were biotin labeled using a membrane impermeable  
741 biotinylation reagent. The cells were shifted with or without antibodies to 37°C during 90 min to  
742 allow endocytosis. Non-internalized biotinylated surface proteins were removed by cleavage with  
743 glutathione, while internalized proteins were protected from biotin removal. After cell lysis,  
744 biotinylated proteins were immunoprecipitated using Streptavidin Mag Sepharose, separated by  
745 sodium dodecyl sulfate-polyacrylamide gel electrophoresis under non-reducing conditions and  
746 detected with RSV F specific antibodies. (A) Sample of non-infected cells. (B) Internalized RSV  
747 F proteins after incubation at 37°C. (C) Internalized RSV F proteins after incubation with RSV F  
748 specific antibodies. (D) Amount of biotinylated surface RSV F proteins after cleavage with  
749 glutathione. (E) Total amount of biotinylated surface RSV F proteins before 37°C incubation  
750 step. A representative blot of a duplicate experiment is shown.

751 **FIG 7 Internalization of Fab fragments.** RSV-infected HEp-2 cells were incubated with RSV F  
752 specific mAbs D25, AM14, 5C4, MPE8 or corresponding monomeric Fab fragments at the same  
753 concentration during 90 minutes to induce internalization. Afterwards the cells were fixed,  
754 permeabilized and stained with AF488 human anti-goat IgG or AF488 chicken anti-mouse IgG  
755 (green). Nuclei were visualized with DAPI (blue). The amount of internalized vesicles was

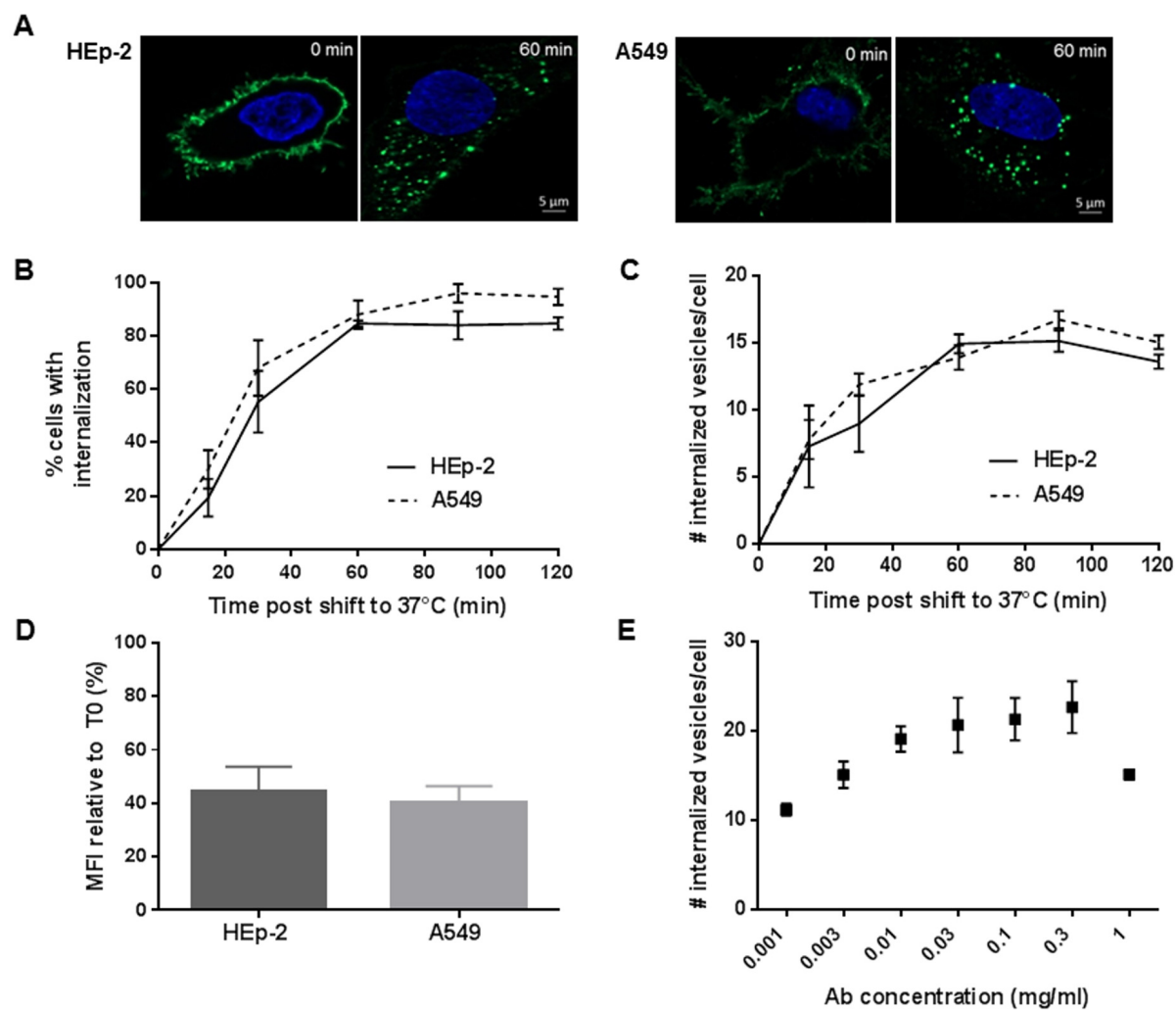
quantified in 50 positive cells. Results are shown as means ( $\pm$ SD) of three independent replicates.  
 (\*,  $P < 0.05$ ).

**TABLE 1 The capacity of different RSV F-specific mAbs to induce internalization.** RSV-infected HEp-2 cells in suspension were incubated with the indicated anti-RSV F mAbs during 1 h at 4°C followed by a shift to 37°C to induce internalization of the attached antibodies. After staining the cells with secondary antibodies, the mean fluorescence intensity (MFI) was measured by flow cytometry. The reduction is expressed as percentage relative to T0 (100%). Data represents the mean of 3 independent repeats.

RSV F-specific mAb	% reduction $\pm$ SD	RSV F antigenic site	RSV F conformation	Reference
131-2A (murine)	31 $\pm$ 9	I	Post ( $\pm$ pre)	(64)
101F (murine)	47 $\pm$ 15	IV	Pre and post	(13)
palivizumab	40 $\pm$ 18	II	Pre and post	(4)
motavizumab	34 $\pm$ 17	II	Pre and post	(65)
AM22	57 $\pm$ 2	Ø	Pre	(14)
D25	50 $\pm$ 16	Ø	Pre	(14)
5C4 (murine)	39 $\pm$ 14	Ø	Pre	(14)
MPE8	44 $\pm$ 9	III	Pre	(15)
AM14	42 $\pm$ 17	V	Pre	(17)

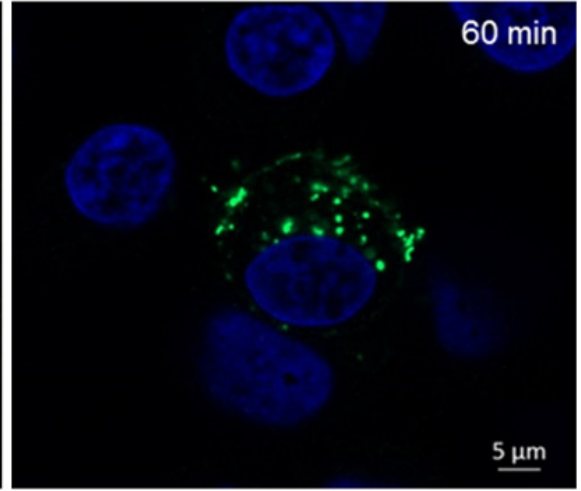
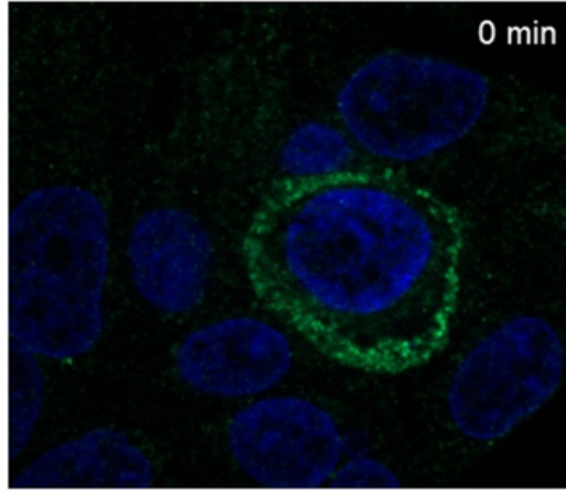
764



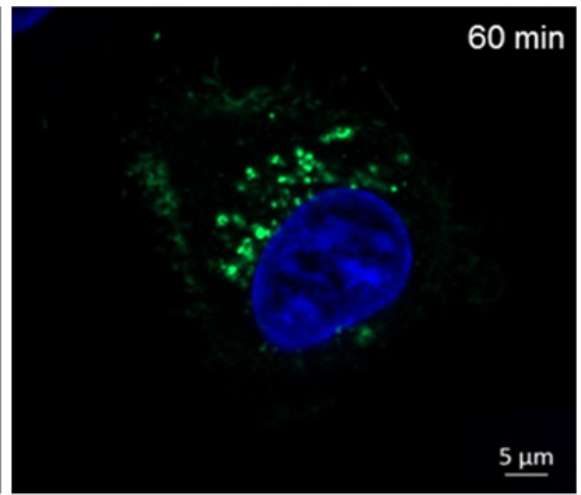
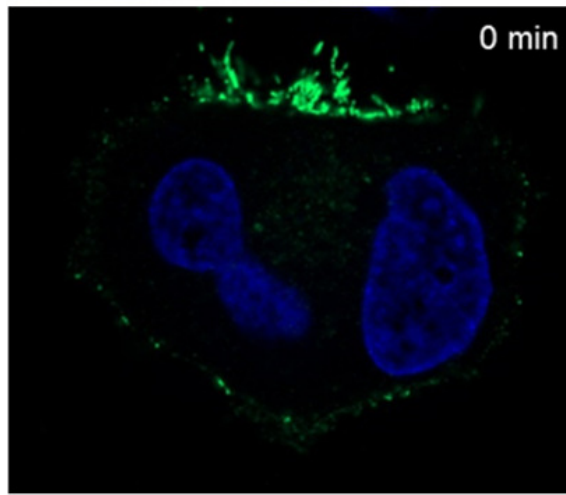


**A**

**B1**



**A1998/3-2**



**B**

